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NOTES

The Primary Structure of *Clostridium septicum* Alpha-Toxin Exhibits Similarity with That of *Aeromonas hydrophila* Aerolysin

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The gene for *Clostridium septicum* alpha-toxin was cloned and expressed in *Escherichia coli* from *C. septicum* BX96. The toxin was determined to be 443 amino acids in length, with a 31-residue signal peptide that was removed from the toxin during secretion. No extended hydrophobic regions were observed in the mature toxin sequence. Expression of alpha-toxin in *E. coli* BL21 resulted in the production of AT^{pro}, which was identical to native toxin from *C. septicum* with respect to activity and activation. The proteolytic activation site for alpha-toxin was determined to be on the carboxy-terminal side of arginine 398, which lies within the sequence KKRRGKR-398SVD. Previous work showing similarities in activation and mechanism between alpha-toxin and *Aeromonas hydrophila* aerolysin was extended to the primary structures of both toxins. The DNA-derived primary sequence of alpha-toxin exhibited 27% identity and 72% similarity over a 387-residue region with the primary structure of the *A. hydrophila* aerolysin toxin, a level of similarity heretofore unobserved between toxins produced by a gram-positive organism and a gram-negative organism.

Clostridium septicum, the causative agent of atraumatic gas gangrene, produces a single lethal factor, alpha-toxin. Bernheimer first described alpha-toxin in 1944 (3), and recently Ballard et al. (1) have isolated and characterized alpha-toxin. On the basis of the report of Ballard et al. (1), alpha-toxin appeared to be the sole lethal factor produced by *C. septicum* and is both lethal to mice and cytolytic. Additional studies (2) into the activity of alpha-toxin have shown that the protein is expressed as a protoxin that requires proteolytic processing for activation. Proteolytic processing results in the removal of an approximately 5-kDa peptide from the carboxy terminus of the protein. Following cleavage of the propeptide, alpha-toxin can oligomerize into a supramolecular complex consisting of six or seven molecules that form ion-permeable channels across cell membranes. The pore size has been estimated to be about 1.5 nm and can allow prelytic release of potassium ions from erythrocytes, suggesting that alpha-toxin-treated cells are lysed via a colloid osmotic process.

Alpha-toxin appears to be unique among clostridia since other clostridial toxins appear to be neither mechanistically nor antigenically similar to alpha-toxin (1). It was noted by Ballard et al. (2) that the activation and mechanism of alpha-toxin resembled that of the cytolytic toxin aerolysin, which is a pore-forming toxin produced by the gram-negative waterborne pathogen *Aeromonas hydrophila* (7, 11, 12). Like alpha-toxin, aerolysin requires carboxy-terminal proteolytic cleavage, resulting in the removal of an approximately 5-kDa peptide for activation. Additionally, the pore-forming aggregates of both toxins consist of five to seven molecules, are resistant to sodium dodecyl sulfate (SDS) dissociation, and form a 1- to 1.5-nm pore in target membranes. As part of this work, the primary structure of alpha-toxin has been deduced from the cloned gene and compared with that of aerolysin. The characteristics of recombinant alpha-toxin expressed in *Escherichia coli* have also been examined and are reported.

Cloning and sequencing of the alpha-toxin gene. *C. septicum* BX96 (a generous gift of Alan Bernheimer) was used as the bacterial source of the chromosomal DNA used in cloning experiments as well as the source of purified alpha-toxin. *C. septicum* BX96 was grown at 37°C in brain heart infusion media containing 0.05% cysteine in a Forma Scientific anaerobic hood. All *E. coli* strains were grown in Luria broth or on Luria-Bertani agar at 37°C with the appropriate antibiotics, when necessary. All media were obtained from Fisher Scientific, and all chemicals were obtained from Sigma unless noted otherwise.

The DNA isolation method developed by Dyer and Iandolo (6) was used to isolate high-molecular-weight chromosomal DNA from *C. septicum.* Oligonucleotides were designed to amplify the alpha-toxin gene on the basis of the deduced DNA sequence from the amino acid sequences of the AT^{pro} amino terminus and the carboxy terminus of the carboxy-terminal cleavage product generated by trypsin activation of AT^{pro} (2). The first primer based on the amino-terminal protein sequence EEGGYANHNN was GAAGAAGGIGGITATGCIAATCA TAATAAT (I, inosine). The second primer, $TCTAAA^{A}_{T}CC^{A}_{T}GG^{A}_{T}ACATCCTG$, was based on the amino-terminal sequence of the trypsin-derived carboxy-terminal propeptide QDVPG FR. A 1-kb PCR product was obtained, ligated into the pCRII cloning vector (50 ng) (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions, and transformed into *E. coli* JM109. A recombinant plasmid (pJB101) containing the

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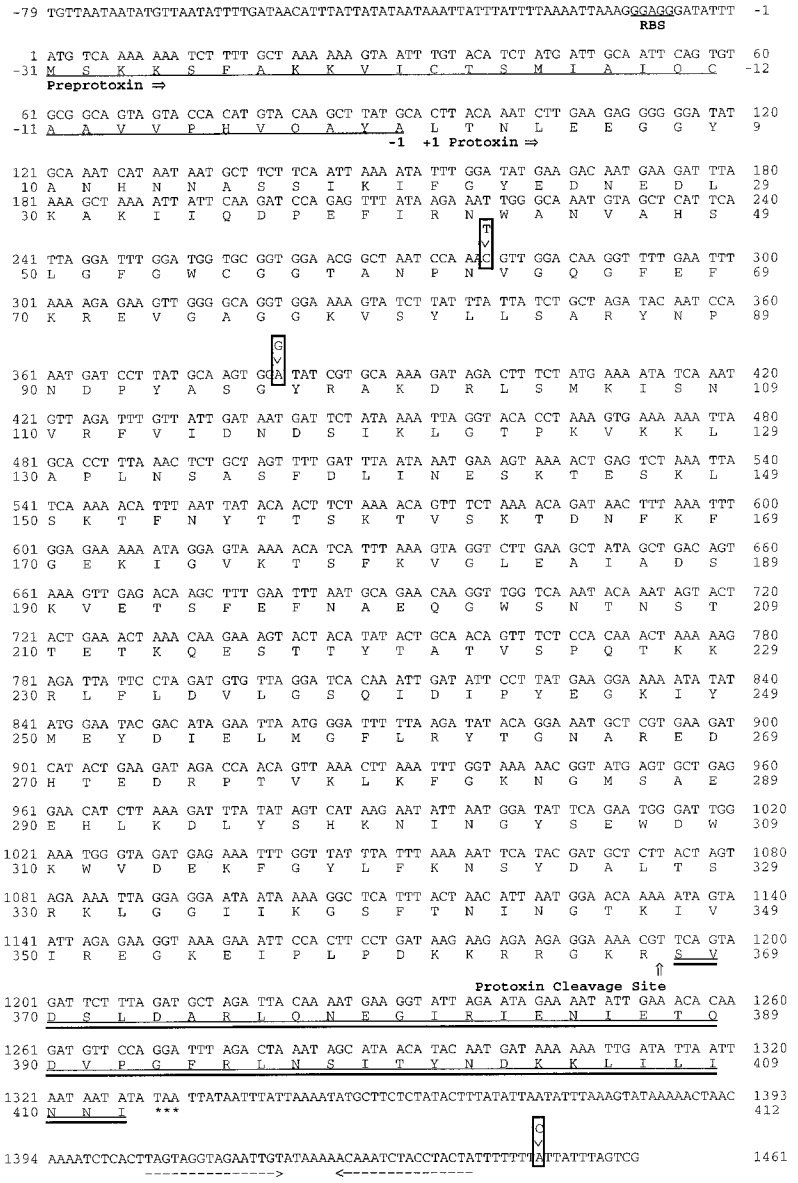

FIG. 1. DNA sequence of the alpha-toxin gene and the primary structure of alpha-toxin. The DNA sequence of the alpha-toxin gene is illustrated along with the DNA-derived primary structure of pre-AT^{pro}. The 31-residue signal peptide is underlined with a single line. The preprotoxin and protoxin start sites as well as the trypsin activation site at Ser-368 are shown; the propeptide is indicated by double underlining. Three nucleotide differences between this sequence and that of Imagawa et al. (13) are boxed; the two nucleotide changes in the coding region of the alpha-toxin protein do not result in amino acid changes. The dashed arrows represent a putative rho-independent transcription terminator. RBS, ribosome binding site.

insert was digested with *Eco*RI, and the insert was gel purified, ligated into *Eco*RI-digested M13mp18, and transformed into *E. coli* JM109. On the basis of the sequence of this clone, primers were designed to amplify the flanking regions of the alpha-toxin gene by inverse PCR. Recombinants from inverse PCR (16) yielded several clones which contained the upstream sequence but not the downstream sequence for alpha-toxin. However, after we had completed approximately 95% of the sequence analysis of the alpha-toxin gene (lacking the sequence of the extreme 3′ end of the gene), the sequence for alpha-toxin was reported by Imagawa et al. (13). On the basis of our sequence data and the sequence of Imagawa et al. (13), two primers (GAAAAAGTATCAGTATTTGAAAAG and ATATAAAAATATCTAGTGTAACGAC) which correspond to residues 247 to 270 and 2020 to 2043, respectively, of the sequence of Imagawa et al. were synthesized. Two independently isolated PCR clones of the alpha-toxin gene were sequenced by automated DNA sequencing (Applied Biosystems, Foster City, Calif.) with fluorescent primers that flanked the polylinker cloning site of pUC18. The alpha-toxin gene was PCR amplified from each clone with primers that flanked the polylinker site of pUC18, PCR products were nebulized (4) to fragment the gene, and fragments were then subcloned into the *Sma*I site of pUC19. Overlapping clones were sequenced, and a 12-fold redundancy was achieved for the entire sequence. No errors due to *Taq* polymerase were detected.

Primary structure of alpha-toxin. The gene sequence for alpha-toxin is shown in Fig. 1. Three nucleotide differences

were observed between our sequence and that of Imagawa et al. (13), none of which resulted in a change in amino acid sequence. As expected, the gene exhibited low GC content (29.3%), which was typical for a clostridial gene. A rho-independent transcription termination terminator was observed 74 bases downstream of the stop codon for alpha-toxin. This repeat and two additional downstream inverted repeats were observed by Imagawa et al. (13).

It was determined that pre-AT^{pro} was 443 amino acids in length (M_r, 49,755), as was reported by Imagawa et al. (13), but on the basis of the amino acid sequence of the first 25 residues of the purified protoxin from *C. septicum* (2), the removal of the signal peptide appeared to occur at the junction of the Ala-32 and Leu-33 (Ala at position −1 and Leu at position +1 on the sequence in Fig. 1) residues, not between Ala-21 and Ala-22 (Ala at positions −10 and −11 in Fig. 1) as suggested by Imagawa et al. (13), on the basis of the rules of von Heijne (21) and the format of Watson (22). Also, if alpha-toxin was processed at Ala-21, the cleavage would occur near the middle of the hydrophobic core of the signal peptide, an unlikely event since no other signal peptide (that we know of) is cleaved within its hydrophobic core. This observation and our previous results which identified the amino terminus of the secreted form of alpha-toxin indicate that alpha-toxin contains a 31-residue signal peptide. The molecular mass of the secreted form (AT^{pro}) was therefore 46,450 Da. In addition, we have shown that AT^{pro} is activated by trypsin cleavage on the amino-terminal side of the sequence SerValAsp (2), and on the basis of the primary structure, this cleavage must occur between Arg-367 and Ser-368 of AT^{pro}.

With the exception of the signal sequence, no extended region of hydrophobicity within the toxin sequence existed. This pattern is similar to that of other aggregating pore-forming toxins (e.g., aerolysin, *Staphylococcus aureus* alpha-toxin, and *C. perfringens* theta-toxin) (8, 9, 14, 15, 18). On the basis of the structure of aerolysin, it appears that hydrophobic surfaces may be generated by beta sheet structures (17) much like those which are observed for porin proteins from the outer membranes of gram-negative bacteria (5). These beta sheets would have an alternating pattern of hydrophobic residues pointed out and hydrophilic residues pointed in and therefore would not appear as a contiguous region of hydrophobic residues upon inspection of the amino acid sequence.

Expression of the alpha-toxin gene in *E. coli*. Although the expression of alpha-toxin in *E. coli* appeared to proceed from its own promoter, its expression was comparatively weak (data not shown). Thus, the coding region of the alpha-toxin gene was fused to the beginning of the *lac* alpha peptide of pUC18 in order to place the expression of alpha-toxin under the control of the *lac* promoter. Two primers GAATTCAAAAAAAA TCTTTTGCTAAAAAAGTA and GGATCCTATAAAATA TCTAGTGTAACGACTA) were designed to juxtapose an *Eco*RI site next to the start codon of the alpha-toxin gene and a *Bam*HI site downstream of the inverted repeat which occurred downstream of the stop codon for alpha-toxin, respectively. This construct fused Ser-2 of alpha-toxin to Asn-6 of the *lac* alpha peptide in pUC18. The resultant plasmid, pRT1000, was transformed into *E. coli* BL21 [F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^- *dcm gal* (DE3)], which is deficient in the LonA and OmpT proteases. Approximately 0.5 to 1 mg of pure alpha-toxin could be isolated from a 1-liter culture of this clone. The expression of alpha-toxin in BL21 resulted in the production of mostly AT^{pro}, as determined by immunoblot (Fig. 2). There are several bands recognized by anti-alpha-toxin which appear to be only in an *E. coli* strain containing pRT1000; however, the major product appeared to be identical in size to wild-type

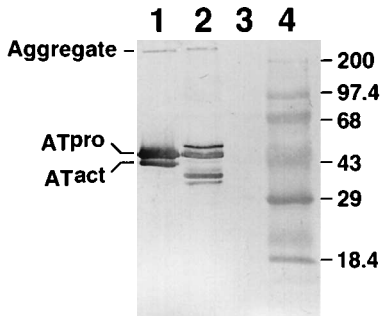

FIG. 2. Immunoblot analysis of recombinant alpha-toxin from *E. coli.* The presence of recombinant alpha-toxin from *E. coli* was determined by separating crude proteins from *E. coli* BL21(DE3)(pRT1000) by SDS-PAGE and then transferring them to nitrocellulose for detection by specific antibody against alpha-toxin. Proteins recognized by the alpha-toxin antibody were from the following sources: lane 1, partially nicked, purified alpha-toxin from *C. septicum* BX96 (proteolytic nicking is caused by an endogenous protease(s) from *C. septicum*); lane 2, lysate proteins from *E. coli* BL21(DE3)(pRT1000); lane 3, lysate proteins from *E. coli* BL21(DE3)(pUC18); and lane 4, molecular weight markers. The positions of AT^{pro}, AT^{act}, and aggregated forms of alpha-toxin are shown on the left.

AT^{pro}. The origin of the band just above the AT^{pro} band in Fig. 2 was not clear; however, it is possible that the larger band may result from read-through of the stop codon for alpha-toxin. This band does not purify with AT^{pro} or appear to be active. No band comigrates with the trypsin-activated form of the toxin (AT^{act}), suggesting that little or no proteolytic processing of AT^{pro} occurs in the periplasm of *E. coli* BL21. The origin of the lower-molecular-weight bands may be due to degradation of the toxin via one or more proteases that do not recognize the RK-rich activation site but may cleave the toxin at other sites. In the lanes containing wild-type and recombinant toxins (lanes 1 and 2 of Fig. 2, respectively), the presence of aggregated toxin is also evident. The aggregation of alpha-toxin in solution was originally observed by Ballard et al. (1) and later shown to be aggregated AT^{act}. The fact that recombinant toxin also aggregates in solution indicates that the recombinant-derived toxin is structurally similar to wild-type toxin.

Purification of recombinant AT^{pro} from *E. coli* BL21(DE3) (pRT1000) was achieved by methods similar to those used for wild-type toxin from *C. septicum* (1) except that *E. coli* cells were initially lysed with a French press to release AT^{pro}. We presumed that the alpha-toxin was secreted by *E. coli* BL21 (DE3) into the periplasm since the signal peptide was removed from purified toxin (see below). However, *E. coli* BL21(DE3) is not amenable to osmotic shock because of *lonA* and *ompT* mutations which apparently affect cell wall maturation; therefore, a clear identification of a periplasmic location for AT^{pro} by cell fractionation methods could not be made. It was determined that the amino-terminal sequence of recombinant alpha-toxin expressed in *E. coli* BL21 was TyrAlaLeuThrAsn-LeuGlu; thus, *E. coli* removed the signal peptide by cleavage between Ala-29 and Tyr-30, instead of between Ala-31 and Leu-32 as is found for *C. septicum.*. The two-residue difference in signal peptide processing site did not appear to alter the hemolytic activity of the recombinant-derived AT^{act}; however, it increases the mass of the recombinant-derived alpha-toxin by 234 Da. Purified recombinant-derived AT^{pro} was similar in size to wild-type AT^{pro} as determined by SDS-polyacrylamide gel electrophoresis (PAGE), (Fig. 3, lanes 2 and 5, respectively). Treatment of the recombinant-derived AT^{pro} with trypsin resulted in the production of a fragment similar in size to AT^{act} isolated from *C. septicum* (Fig. 3, lanes 3 and 4, respectively).

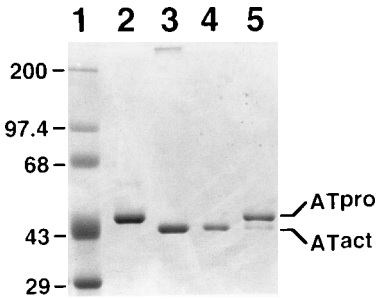

FIG. 3. Purification and trypsin activation of recombinant alpha-toxin. Alpha-toxin from *E. coli* BL21(DE3) was purified and treated with trypsin. Shown is the SDS-PAGE analysis of AT^{pro} and AT^{act} purified from *C. septicum* and *E. coli.* Lane 1, molecular weight markers; lane 2, AT^{pro} from *E. coli;* lane 3, trypsin-treated AT^{pro} from *E. coli;* lane 4, trypsin-treated alpha-toxin from *C. septicum*; lane 5, alpha-toxin from *C. septicum* (AT^{pro} from *C. septicum* is partially cleaved by endogenous protease).

The hemolytic activity of the recombinant-derived AT^{act} was nearly identical to that of AT^{act} generated by trypsin cleavage of AT^{pro} from *C. septicum* (approximately 1×10^6 to 10×10^6 hemolytic units/mg).

Similarity of alpha-toxin to aerolysin. The primary structure of alpha-toxin confirmed our original suggestion (2) that the similarities in the activation and cytolytic mechanisms of alpha-toxin and aerolysin might be reflected in similarities in the primary structures of these two toxins. Imagawa et al. (13) reported that they did not find sequence similarity with any other protein; however, we found that the primary structure of the *A. hydrophila* proaerolysin toxin exhibited more than 27% identity with *C. septicum* AT^{pro} over a contiguous region of 387 amino acids (Fig. 4). In addition, if conservative substitutions are considered, these toxins exhibit approximately 72% similarity.

The similarity between alpha-toxin and aerolysin is the first example (that we know of) of this level of sequence similarity between the primary structures of toxins produced by a gram-positive bacterial species and a gram-negative bacterial species. The fact that the G+C content of the alpha-toxin gene is almost exactly one-half (29.3%) of that of the aerolysin gene (58.6%) suggests that these two genes have had sufficient time for considerable divergence of the gene sequence. The extended sequence similarity in the primary structures of these two toxins indicates that divergent evolution is responsible for the observed similarity. The similarity between AT^{pro} and aerolysin starts at residue 25 of AT^{pro} and residue 97 of aerolysin and continues almost to the carboxy terminus of each molecule. On the basis of the crystal structure of aerolysin (17), the similarity extends throughout domains 2 to 4 of the aerolysin crystal structure. A notable difference in the primary structures of these two toxins is that AT^{pro} lacks sequence similarity with the first 96 residues of proaerolysin. Overall, aerolysin has 72 more residues at its amino terminus than does AT^{pro} and these residues largely constitute domain 1 of aerolysin. The apparent absence of a homolog in AT^{pro} for domain 1 of aerolysin suggests that it encodes a special function for aerolysin.

Similarities also existed for conspicuous features of each toxin; for instance, the tryptophan-rich sequence of Trp-307AspTrpLysTrp in alpha-toxin corresponds in position to the tryptophan-rich sequence of Trp-371AspTrpAsnTrp in aerolysin. Van der Goot et al. (20) have shown that Trp-371 and Trp-373 have been implicated in the oligomerization process of aerolysin. The proteolytic activation sites for alpha-

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aerolysin      1 AEPVYPDQLRLFSLGQGVCGDKYRPVNREEAQSVKSNIVGMMGQWQISGL

alpha toxin    1 ----------------------LTNLEEGGYANHNNASSIKIFGYEDNED
                                                                :. :
aerolysin     51 ANGWVIMGPGYNGEIKPGTASNTWCYPTNPVTGEIPTLSALDIPDGDEVD

alpha toxin   29 LKAKIIQDP-EFIRNWANVAHSLGFGWCGGTANPNVGQGFEFKREVGAGG
                  .. ....:. .::.  . .:: ::..: ::   .  ::.. .  :. ..
aerolysin    101 VQWRLVHDSANFIKPTSYLAHYLGYAWVGGNHSQYVGEDMDVTRDGDG--

alpha toxin   78 KVSYLLSARYNPNDPYASGYRAKDRLSMKISNVRFVIDNDSIKLG--TPK
                    .. .    ::    :::  :. ..:.::   . .: ::.: :  :
aerolysin    149 ---WVIRGN---NDGGCDGYRCGDKTAIKVSNFAYNLDPDSFKHGDVTQS

alpha toxin  126 VKKLAPLNSASFDLINESKTESKLSKTFNYTTSKTVSKTDNFKFGEKIGV
                  ..:      .  . .    .:    :. : :.   :::. . ..::.
aerolysin    193 DRQLVK-TVVGWAVNDSDTPQSGYDVTLRYDTATNWSKTNTYGLSEKVTT

alpha toxin  176 KTSFKVGLEAIADSKVETSFEFNAEQGWSNTNSTTETKQESTTYTATVSP
                 :  ::  :  .... .  :.:. :.:.:.. :. . :   : .  .::..
aerolysin    242 KNKFKWPL--VGETEL--SIEIAANQSWASQNGGSTTTSLSQSVRPTVPA

alpha toxin  226 QTKKRLFLDVLGSQIDIPYEGKIYMEYDIELMGFLRYTGNAREDHTEDRP
                 ..:  . ...  ..:  ::: :  . ::. : ::::  :::.  : ..::
aerolysin    288 RSKIPVKIELYKADISYPYEFKADVSYDLTLSGFLRWGGNAWYTHPDNRP

alpha toxin  276 TVKLKFGKNG-MSAEEHLKDLYSHKNINGYSE-WDWKWVDEKFGYLFKNS
                   .  :           ..     . : :    :::.:  .. :    ..
aerolysin    338 NWNHTFVIGPYKDKASSIRYQWDKRYIPGEVKWDWNWTIQQNGLSTMQN

alpha toxin  324 YDALTSRKLGGIIKGSFTNINGTKIVIREGKEIPLP-DKKRRGKRSVESL
                   :   : . . : : :.  .     :  :  .::. : : :  ::::.
aerolysin    388 NLARVLRPVRAGITGDFSAESQFAGNIEIGAPVPLAADSKVRRARSVDG-

alpha toxin  373 DARLQNEGIRIE-NIETQDVPGFRLNSITYNDKKLILINNI
                        .:.:.  ...:...:. .:... .   .    :
aerolysin    437 ----AGQGLRLEIPLDAQELSGLGFNNVSLS---VTPAANQ
```

FIG. 4. Similarity between alpha-toxin and aerolysin. The primary structures of AT^{pro} and aerolysin were aligned by using Clustal V (10). The amino acid sequences of only the secreted forms of each protoxin were aligned. Single dots indicate conservative substitutions, and double dots signify conserved residues. AT^{pro} exhibits 27% identity and 72% similarity with aerolysin over 387 residues. The tryptophan-rich region and protoxin cleavage are enclosed by boxes.

toxin and aerolysin also exhibited sequence and positional similarities. As mentioned above, the cleavage of AT^{pro} occurred between residues Arg-367 and Ser-368; however, Arg-367 is juxtaposed to an upstream region that is RK rich (Fig. 1). The abundance of arginine and lysine residues makes this site particularly susceptible to proteases that recognize basic amino acids and so it is possible that AT^{pro} may be preferentially cleaved at one of these other Arg or Lys residues, depending on the protease specificity. The Arg-368SerValAsp sequence in aerolysin is also conserved and juxtaposed to a lysine and arginine-rich sequence. Aerolysin is activated by trypsin cleavage at Lys-427 and by chymotrypsin cleavage at Arg-429 (19), both of which are juxtaposed to the Arg-432SerValAsp sequence of aerolysin. The cleavage of alpha-toxin on the carboxy side of Arg-367 by trypsin activates alpha-toxin and generates a carboxy-terminal peptide of 45 amino acids with a molecular weight of 5,140. It is not yet known if the 5,140-Da peptide remains associated with the large, cytolytically active fragment of the toxin (AT^{act}) or is released upon cleavage. We had originally suggested that this propeptide functions as an intramolecular chaperone sequence (2); however, we cannot rule out the possibility that the propeptide participates in the cytolytic process or has some other function in vivo after its release from AT^{pro}. The site of this cleavage, RGKR-367 ↓

SVD, precisely fits a furin consensus recognition site of RXKR. Whether furin plays a role in the activation of alpha-toxin remains to be determined, although it seems likely that many different proteases which recognize Arg or Lys could potentially activate the toxin in vivo as they do in vitro (2). The analogous cleavage site in aerolysin is presumed to be in a solvent-exposed flexible loop at the tip of domain 4 of the aerolysin molecule; the position of these residues could not be accurately resolved in the aerolysin crystal, suggesting that this region is highly flexible (17).

The study of alpha-toxin, though in its infancy, has proven to be interesting from both mechanistic and evolutionary aspects. Currently, work is proceeding to crystallize this toxin to determine if the tertiary structure of AT^{pro} resembles that of proaerolysin. Also, questions remain concerning the transition of alpha-toxin from the aqueous milieu to the cell membrane; the nature of the molecular events which lead up to the insertion of these hydrophilic molecules into membranes remains unknown.

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REFERENCES

1. **Ballard, J., A. Bryant, D. Stevens, and R. K. Tweten.** 1992. Purification and characterization of the lethal toxin (alpha-toxin) of *Clostridium septicum.* Infect. Immun. **60:**784–790.
2. **Ballard, J., Y. Sokolov, W.-L. Yuan, B. L. Kagan, and R. K. Tweten.** 1993. Activation and mechanism of *Clostridium septicum* alpha toxin. Mol. Microbiol. **10:**627–634.
3. **Bernheimer, A. W.** 1944. Parallelism in the lethal and hemolytic activity of the toxin of *Clostridium septicum.* J. Exp. Med. **80:**309–320.
4. **Bodenteich, A., S. Chissoe, Y. F. Wang, and B. A. Roe.** 1994. Shotgun cloning as the strategy of choice to generate templates for high-throughput dideoxynucleotide sequencing, p. 42–50. *In* M. D. Adams, C. Fields, and J. C. Venter (ed.), Automated DNA sequencing and analysis techniques. Academic Press, London.
5. **Cowan, S. W., T. Schirmer, G. Rummel, S. Steiert, R. Ghosh, R. A. Pauptit, J. N. Jansonius, and J. P. Rosenbusch.** 1992. Crystal structures explain functional properties of two *Escherichia coli* porins. Nature (London) **358:** 727–733.
6. **Dyer, D. W., and J. J. Iandolo.** 1983. Rapid isolation of DNA from *Staphylococcus aureus.* Appl. Environ. Microbiol. **46:**283–285.
7. **Garland, W. J., and J. T. Buckley.** 1988. The cytolytic toxin aerolysin must aggregate to disrupt erythrocytes, and aggregation is stimulated by human glycophorin. Infect. Immun. **56:**1249–1253.
8. **Geoffroy, C., J. Mengaud, J. E. Alouf, and P. Cossart.** 1990. Alveolysin, the thiol-activated toxin of *Bacillus alvei,* is homologous to listeriolysin O, perfringolysin O, pneumolysin, and streptolysin O and contains a single cysteine. J. Bacteriol. **172:**7301–7305.
9. **Haas, A., M. Dumbsky, and J. Kreft.** 1992. Listeriolysin genes: complete sequence of ILO from *Listeria ivanovii* and of ISO from *Listeria seeligeri.* Biochim. Biophys. Acta **1130:**81–84.
10. **Higgins, D. G., and P. M. Sharp.** 1988. Clustal: a package for performing multiple sequence alignment on a microcomputer. Gene **73:**237–244.
11. **Howard, S. P., and J. T. Buckley.** 1982. Membrane glocoprotein receptor and hole-forming properties of a cytolytic protein. Biochemistry **21:**1662–1667.
12. **Howard, S. P., and J. T. Buckley.** 1985. Activation of the hole-forming toxin aerolysin by extracellular processing. J. Bacteriol. **163:**336–340.
13. **Imagawa, T., Y. Dohi, and Y. Higashi.** 1994. Cloning nucleotide sequence and expression of a hemolysin gene of *Clostridium septicum*. FEMS Microbiol. Lett. **117:**287–292.
14. **Kehoe, M. A., L. Miller, J. A. Walker, and G. J. Boulnois.** 1987. Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. Infect. Immun. **55:**3228–3232.
15. **Mengaud, J., M.-F. Vicente, J. Chenevert, J. M. Pereira, C. Geoffroy, B. Gicquel-Sanzey, F. Baquero, J.-C. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolsyin O determinant of *Listeria monocytogenes.* Infect. Immun. **56:**766–772.
16. **Ochman, H., A. S. Gerber, and D. L. Hartl.** 1988. Genetic applications of an inverse polymerase chain reaction. Genetics **120:**621–623.
17. **Parker, M. W., J. T. Buckley, J. P. M. Postma, A. D. Tucker, K. Leonard, F. Pattus, and D. Tsernoglou.** 1994. Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. Nature (London) **367:**292–295.
18. **Tweten, R. K.** 1988. Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens:* significant homology with the genes for streptolysin O and pneumolysin. Infect. Immun. **56:**3235–3240.
19. **Van der Goot, F. G., J. Lakey, F. Pattus, C. M. Kay, O. Sorokine, A. V. Dorlasselaer, and J. T. Buckley.** 1992. Spectroscopic studies of the activation and oligomerization of the channel-forming toxin aerolysin: identification of the site of proteolytic activation. Biochemistry **31:**8566–8570.
20. **Van der Goot, F. G., F. Pattus, K. R. Wong, and J. T. Buckley.** 1993. Oligomerization of the channel-forming toxin aerolysin precedes insertion into lipid bilayers. Biochemistry **21:**2636–2642.
21. **von Heijne, G.** 1984. How signal sequences maintain cleavage specificity. J. Mol. Biol. **173:**243–251.
22. **Watson, M. E. E.** 1984. Compilation of published signal sequences. Nucleic Acids Res. **12:**5145–5164.